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FOREWORD

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Introduction

Undifferentiated lymphomas similar to Burkitt's lymphoma are frequent in patients with acquired immunodeficiency syndrome (AIDS).¹⁻⁷ Many of these lymphomas occurring in AIDS patients contain Epstein-Barr virus (EBV) and display characteristic chromosomal translocations.^(2,6,8-11) Chronic lymphocytic interstitial pneumonitis, another lymphoproliferative disease associated with EBV, has also been reported at high frequency in HIV infected patients, particularly in children.⁽¹²⁾

In previous studies we have found that patients with HIV infection generally display abnormally elevated numbers of EBV-infected B cells in the circulation.⁽¹³⁾ These virally infected, continuously cycling B cells may predispose HIV-infected individuals to the development of EBV associated lymphoproliferations.

EBV is a ubiquitous herpesvirus with oncogenic potential. In vitro, the virus transforms B cells into continuously proliferating, immortal cell lines.⁽¹⁴⁾ Most normal adults have a small number of EBV-infected B cells in the circulation with a potential for continuous proliferation. However, EBV-related lymphoproliferations are rare in man.^(15,16) An array of immune regulatory mechanisms, including T cell killing and T cell suppression,¹⁷⁻¹⁹⁾ are believed to play an important role in the control of natural EBV infection.

The present studies were designed to understand the basis for dysregulation of EBV infection in HIV-infected individuals.

Body

A. Growth of EBV-infected B cells: Requirements

To best study the basis for dysregulation of EBV-infection in HIV-infected individuals resulting in abnormally elevated numbers of EBV-infected cells in the circulation, the molecular mechanisms of B cell growth induced by EBV and their regulation had to be understood. Recently, it was observed that the continuous proliferation of EBV-infected B cells is dependent, at least in part, upon the presence of growth factors that are produced by the same virally infected cells (autocrine growth factors).⁽²⁰⁻²²⁾ This observation has suggested that immortalization by EBV may require that the virus induce B cells to secrete growth factors and to express appropriate receptors for these molecules.⁽²³⁾ Although several studies have analyzed the nature of autocrine growth factors produced by EBV-induced lymphoblastoid cell lines, the molecular nature of these growth factor or factors still remains controversial.

Three molecules have been implicated: "B cell growth factor (Bcgf)," interleukin-1 (IL-1) and a soluble form of the B cell activation antigen CD23.⁽²⁴⁻²⁹⁾ We have analyzed each of these possibilities in great detail. First, we have established an in vitro assay system that allows us to measure effectively growth stimulation of EBV-infected cells when exposed to exogenous growth factors. In this system, exponentially growing EBV-immortalized B cells are cultured in a starvation medium for 24 h that allows the cells to survive but not to proliferate. Starved

B cells are then cultured at low cell densities in nutrient poor medium with or without exogenous growth factors. Under these conditions, EBV immortalized B cells proliferate very little in medium alone and display a brisk proliferative response when exposed to autologous culture supernatants obtained during exponential growth (Fig. 1). Using this assay system, we have conclusively established that neither IL-4 (a T cell derived Bc_gf) (not shown) nor IL-1 α or β (Fig. 2), nor soluble CD23 (Fig. 3) are the molecules responsible for autocrine growth stimulation in the EBV-immortalized cell lines we have tested. IL-4 as well as IL-1 α and β used in these assays were highly purified recombinant preparations which displayed the appropriate biological properties attributed to them. CD23 was affinity purified from B line culture supernatants. Identification of this affinity purified material as CD23 was obtained both by SDS-PAGE (Fig. 4) as well as by a specific ELISA assay (Fig. 5).

Because the molecules previously reported to act as autocrine growth factors for EBV-immortalized B cells failed to display this property when tested upon a panel of EBV-immortalized B cells, we concluded that, at least for these lines, autocrine growth stimulation involves other molecules. We set out to identify these molecules.

First, we have identified a high producer EBV-immortalized cell line, called V). Subsequently, we have devised culture conditions which favor autocrine growth factor production in serum-free culture medium. Optimal production (16-32 U/ml) was

found to occur when exponentially growing cells are washed free of serum and cultured for 18 h with BSA (1 mg/ml) and transferrin (2.5 μ g/ml). Under these conditions, we have identified two autocrine growth factors which display distinct molecular mass. A high molecular weight factor that elutes from gel filtration columns (Sephadex G-75) with a relative mass of approximately 60-70 kDa (Fig. 6), and a low molecular weight factor elutes from gel filtration columns (P2) with a relative mass of 1000-500 Da (Fig. 7). Because of its small size, uncommon among previously described growth factors, we have focused our attention on the low molecular weight autocrine growth factor. Over the last several months we have devised a number of purification steps that have permitted to purify significantly this low molecular weight factor from contaminating molecules. Final chemical characterization of this molecule requires further purification, followed by sequencing and/or, where appropriate, mass spectrometry and nuclear magnetic resonance.

In addition to these efforts toward its chemical characterization, the low molecular weight growth factor in semi-purified form has been characterized from a biological point of view, particularly for its B cell growth promoting potential. Interestingly, it does not induce growth in B cells activated by polyclonal activators such as *Staphylococcus aureus* cowan 1 (SAC) and pokeweed mitogen, while it greatly augments the growth of EBV-infected B cells (Table 1). This suggests selectivity for

certain target B cell and/or selectivity for certain types/stages of B cell activation.

In related experiments, we have examined the possibility that factors other than those produced by EBV-immortalized B cells might also promote growth of EBV-infected B cells. Supernatants of activated peripheral blood monocytes were found to greatly augment growth in EBV-infected B cells. By contrast, supernatants of activated T cells failed to do so.^(30,31) The active molecule was purified from these monocyte-derived supernatants, and identified as IL-6.⁽³¹⁾

Is IL-6 also produced by EBV-immortalized B cells? Several cell types have been reported to secrete IL-6 including fibroblasts, monocytes, endothelial cells, and keratinocytes.⁽³¹⁾ However, B cells are not believed to express the IL-6 gene. In recent experiments, we have determined that EBV-immortalized B cells cultured under conditions that maximize growth factor production, fail to express the IL-6 gene (not shown), as determined by Northern blotting. In addition, supernatants of lymphoblastoid cell lines containing large quantities of autocrine growth factors, do not contain IL-6, as determined by immunoprecipitation (Fig. 8) and Western blotting (not shown). Thus, IL-6 is a paracrine growth factor that promotes growth in EBV-infected B cells.

Taken together, these findings support the notion that continuous proliferation of EBV-immortalized B cells is dependent upon the presence of growth factors. These factors include

autocrine factors, both a high and a low molecular weight component, and a paracrine growth factor, IL-6.

B. Dysregulation of EBV infection in HIV-infected individuals

Previous studies in the laboratory have demonstrated that patients with AIDS or AIDS-related complex have abnormally high numbers of EBV-infected B cells in the circulation.⁽¹³⁾ In vitro, several features characterize immune dysfunction in HIV-infected patients. These include the presence of abnormally high numbers of B cells spontaneously secreting Ig, indicating the presence of circulating activated cells.⁽¹³⁾ This spontaneous B cell activation can be explained only in part by the increased number of EBV-infected B cells in the circulation.⁽³⁴⁾ When infected in vitro with EBV, B lymphocytes from HIV-infected individuals proliferate and produce Ig at a significantly lower rate than normals (not shown). This is probably due to the fact that a large proportion of these B cells are preactivated in vivo, and it is known that only resting B cells can be induced to grow by EBV.

Normal EBV-seropositive T cells have been shown to markedly suppress in vitro autologous B cells experimentally infected with exogenous EBV.⁽³⁵⁾ By contrast, when tested under identical conditions, T cells from EBV seropositive HIV-infected patients fail to suppress autologous EBV-infected B cells.⁽¹³⁾ Taken together, these studies suggest that the existence of elevated numbers of EBV-infected B cells in the blood of HIV-infected

patients is probably due to ineffective EBV-related T cell regulation in these individuals.

Why are CD8 positive EBV-related regulatory T cells defective in HIV-infected patients? In many of these patients the number of CD8 positive T cells is normal. When tested for their capacity to generate non-specific suppressor T cells upon Con A stimulation, T cells from HIV positive individuals were not significantly different from normal T cells (not shown).

An alternative possibility is that EBV-infected B cells from these patients are more difficult to suppress. In this respect, we have examined whether growth factors known to favor growth of EBV-infected B cells are more abundant in HIV-infected individuals. As mentioned above, we have identified IL-6 as one such monocyte-derived growth factor. Therefore, we have now tested whether HIV-infected individuals have higher levels of serum IL-6 compared to normals. As shown in Table 2, HIV-infected individuals, particularly those with stage 1 plus 2 disease, have significantly higher levels of serum IL-6 compared to normals. Furthermore, supernatants of monocyte cultures from HIV-positive individuals produce spontaneously significantly higher levels of IL-6 compared to normal monocyte cultures (Table 3). This finding is supported by the experimental observation that HIV is a potent inducer of IL-6 in human normal monocytes (not shown). Thus, abnormally high levels of serum IL-6 in HIV-positive individuals is responsible, in part, for the associated existence of abnormally elevated numbers of EBV-infected B cells.

Similarly, abnormally elevated levels of IL-6 in culture might prevent EBV-related suppressor T cells from suppressing autologous EBV-infected B cells.

Conclusions

Our efforts have focused on a better understanding of the molecular basis for B cell growth induced by EBV and how this process is dysregulated in HIV-positive individuals. Advances in our understanding of the requirements for B cell immortalization by EBV have permitted to better define immune dysfunction related to EBV in HIV-positive individuals.

The experiments described here have demonstrated that long-term growth of EBV-infected B cells is strikingly dependent upon availability of growth factors. Some of these growth factors are produced by the same virally infected cells and are therefore called autocrine, others are not produced by the virally infected cells and are therefore called paracrine. We have identified two autocrine growth factors produced by EBV-immortalized B cells; a 60-70 kDa protein and a peptide of approximately 1000-500 Da. In addition, we have identified IL-6, a monocyte-derived growth factor, as a paracrine stimulator of growth in EBV-infected B cells.

Patients with AIDS and AIDS-related disorders have abnormally elevated numbers of EBV-infected B cells in the circulation. In vitro, T cells from these patients are defective in their ability to suppress autologous B cells infected in vitro with exogenous EBV. In these patients serum levels of IL-6 are

abnormally elevated compared to normals. In vitro, monocyte cultures from HIV-positive individuals secrete spontaneously much greater amounts of IL-6 compared to normal monocyte cultures. Abnormally elevated levels of IL-6 in HIV-positive individuals may be responsible for the abnormally elevated numbers of circulating EBV-infected B cells in these individuals, because we have found that in vitro this is one of the biological effects of IL-6. Furthermore, abnormally high secretion of IL-6 by monocytes in culture may explain why T cells from HIV-positive individuals fail to suppress autologous EBV-infected B cells stimulated to grow by IL-6.

Future work will focus on the molecular identification of the two autocrine growth factors produced by EBV-infected B cells described above. For the high molecular weight growth factor gene cloning will be pursued. When these factors will be identified and their precise role for B cell immortalization will be clarified, we will examine their production, function and regulation in HIV-infected individuals.

Growth factors are emerging as essential for growth of EBV-infected B cells and their regulation. Clarification of their molecular nature, function and production during HIV infection will help in further understanding EBV infection in HIV infected individuals. In addition, understanding growth factor dysregulation during HIV infection may help in better understanding this viral illness.

Table 1

Effects of a low molecular weight growth factor on B cell activation

Cell culture	Additives	Proliferation cpm/culture	
		Exp 1	Exp 2
B cells ^a	medium	184	68
	low mw factor 8 U	192	103
	4 U	202	104
	2 U	154	92
SAC-activated B cells	medium	649	1,250
	Namalwa factor	3,109	4,953
	low mw factor 8 U	641	1,112
	4 U	519	845
	2 U	582	784
PWM-activated B cells	medium	3,147	1,896
	irradiated T cells	81,382	45,714
	low mw factor 8 U	2,772	1,150
	4 U	2,779	954
	2 U	1,744	1,122
EBV-B cells	medium	174	88
	IL-6 10 U/ml	3,412	3,053
	low mw factor 8 U	6,140	6,545
	4 U	4,322	2,564
	2 U	1,210	561

^aB cells, SAC-activated B cells, PWM-activated B cells were cultured at 50×10^3 /well; EBV-infected B cells from a lymphoblastoid cell line were cultured at 3×10^3 cells/well.

^bcpm/culture; ³H was added during the final 18 h of a 3 day culture.

Table 2

Serum IL-6 levels are abnormally elevated in HIV-infected individuals

Test population	Sample number	Serum IL-6 levels (pg/ml)
Normal	10	3 ± 4^a
HIV-infected stage 1/2	10	281 ± 183
HIV-infected stage 3/4	10	176 ± 159
HIV-infected stage 5/6	10	96 ± 113

^a IL-6 levels were compared to a standard IL-6 preparation.

Table 3

High rate IL-6 secretion in long-term monocyte cultures from HIV-positive individuals

Test cultures	Secreted IL-6 (pg/ml)	
Normal monocyte cultures		
sample 1	32 ^a	
2	34	
3	29	
4	368	
mean value		116 ± 168
HIV-positive monocyte culture		
sample 1	320	
2	4914	
3	3890	
4	1536	
mean value		2665 ± 2108

^aIL-6 levels are compared to a standard IL-6 preparation.

**SUPERNATANT OF EBV-IMMORTALIZED B CELLS HAS
AUTOCRINE GROWTH FACTOR ACTIVITY**

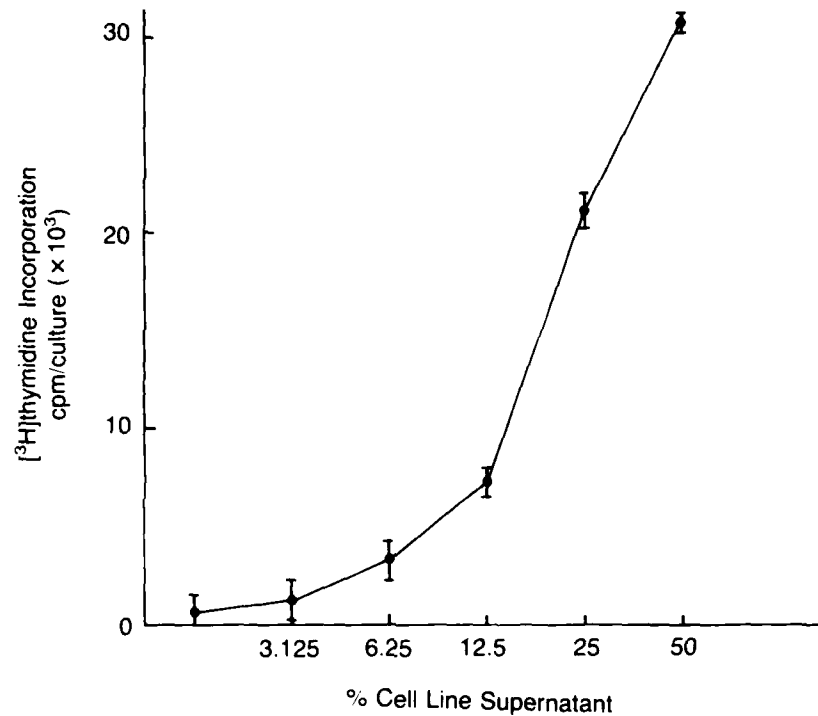


Figure 1. EBV-immortalized B cells proliferate in response to autocrine growth factors. Starved cells from a lymphoblastoid cell line (V) were cultured at low cell density (3×10^3 cells/well) in medium supplemented with varying concentrations of autologous supernatant.

RECOMBINANT IL-1 β FAILS TO STIMULATE GROWTH IN EBV-IMMORTALIZED B CELLS

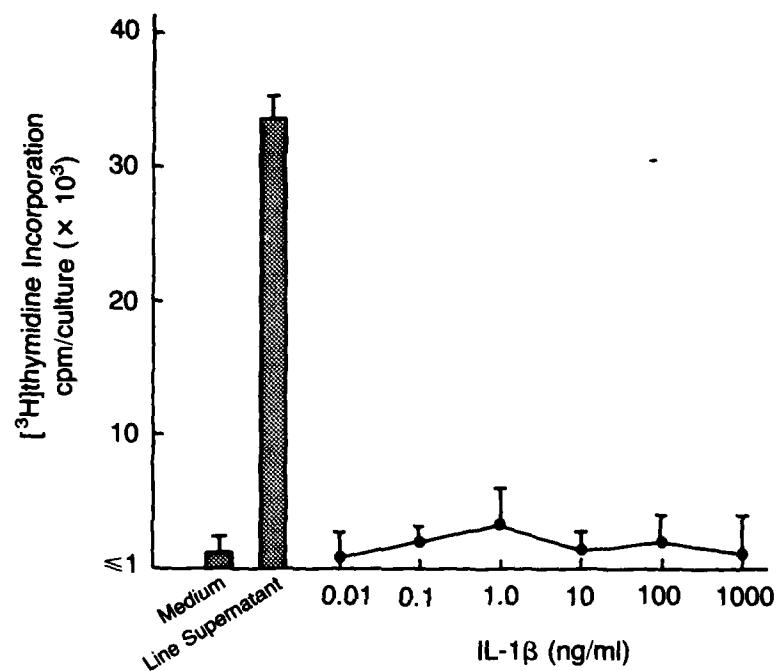


Figure 2. Failure of IL-1 to stimulate growth in EBV-immortalized B cells. Starved cells from an EBV-immortalized B cell line were cultured in either medium alone or medium supplemented with recombinant IL-1 at varying concentrations.

AFFINITY PURIFIED SOLUBLE CD23 FAILS TO PROMOTE GROWTH IN EBV-IMMORTALIZED B CELLS

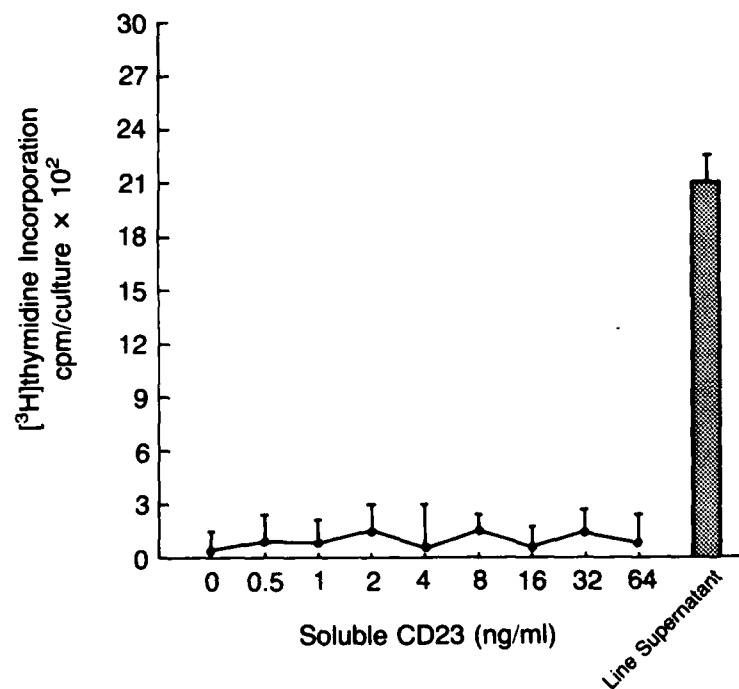


Figure 3. Failure of affinity purified soluble CD23 to stimulate growth in EBV-immortalized B cell lines. Starved EBV-immortalized B cells were cultured either in medium alone or supplemented with affinity purified soluble CD23.

AFFINITY PURIFIED SOLUBLE CD23 SILVER STAIN

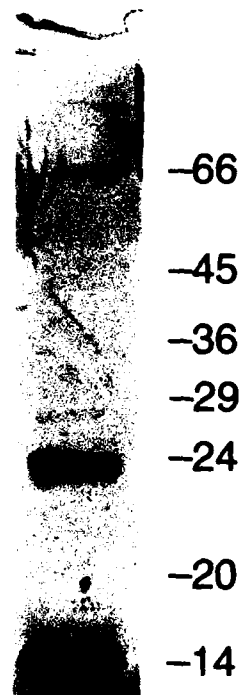


Figure 4. Polyacrylamide gel electrophoresis of affinity purified CD23. Soluble CD23 was purified by affinity from the culture supernatant of an EBV-immortalized B cell line using an anti-CD23 monoclonal antibody (CS-5) bound to Cn Bromide-activated Sepharose 4B and analyzed by electrophoresis on a 1% SDS-12% polyacrylamide gel. After electrophoresis, the gel was stained by silver.

TESTING OF AFFINITY PURIFIED SOLUBLE CD23

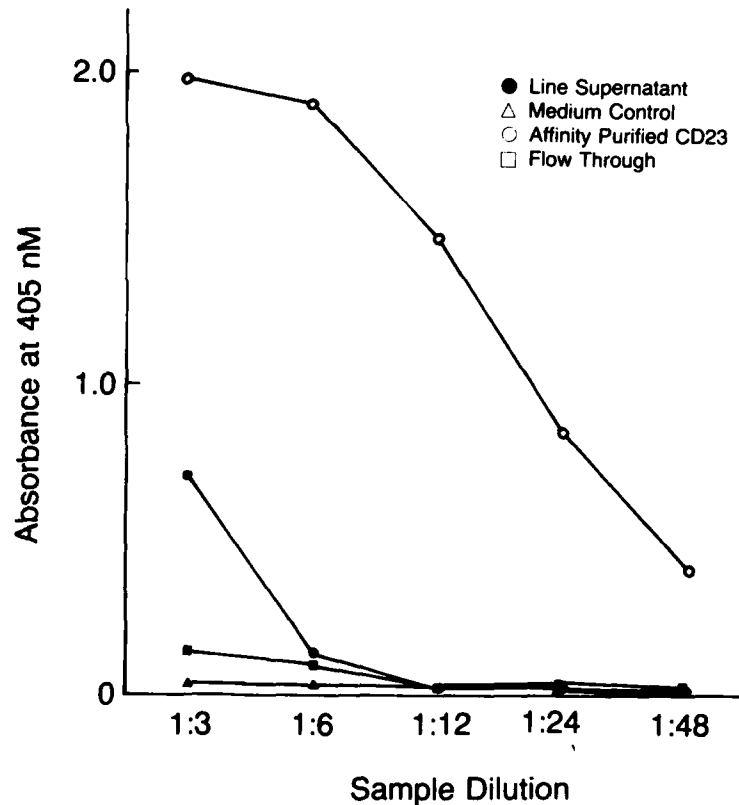


Figure 5. Affinity purified soluble CD23 reacts with anti-CD23 monoclonal antibodies in an ELISA. Microtiter plates were coated with an anti-CD23 monoclonal antibody (CS-5; IgG class at 5 μ g/ml); test samples, serially diluted, were incubated in the anti-CD23 coated plates; an anti CD-23 monoclonal antibody (CS-4; IgM class purified by precipitation with ammonium sulfate, used at 1:100 dilution) was added to the plates; goat anti-mouse IgM alkaline phosphatase conjugated (Cappel - 40 μ g/ml) followed by a substrate was used as developing reagent.

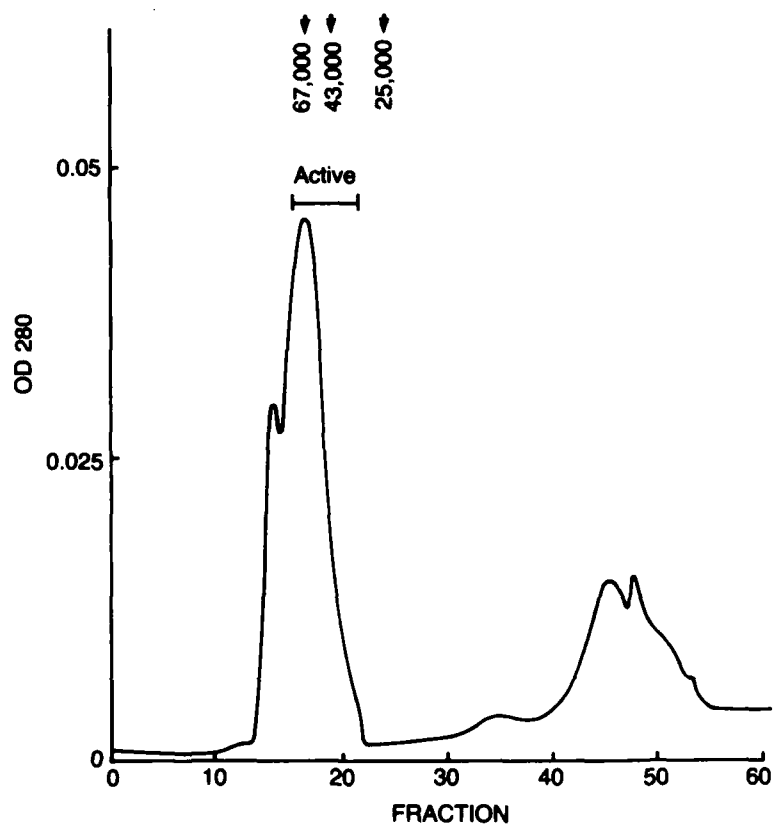


Figure 6. Determination of the relative molecular mass of an autocrine growth factor produced by the EBV-infected lymphoblastoid cell line (V). Gel filtration over Sephadex G-75 (Pharmacia).

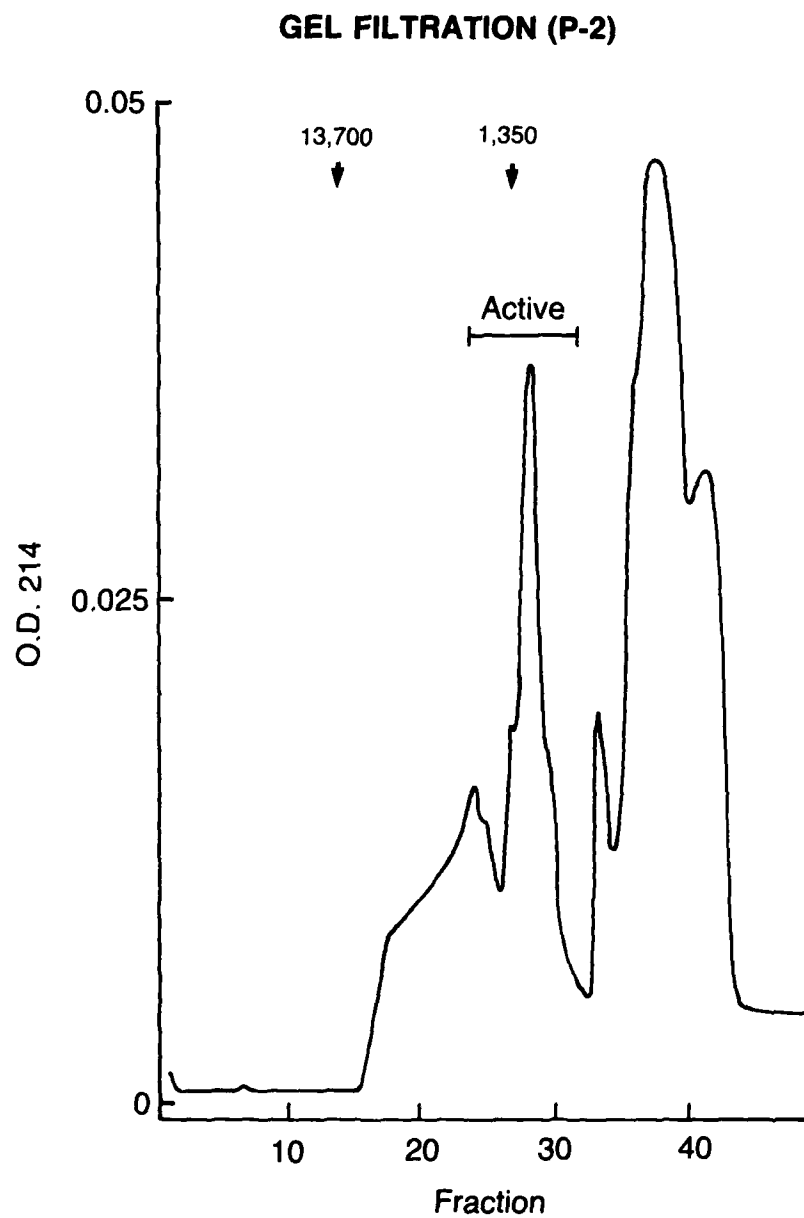


Figure 7. Determination of the relative molecular mass of an autocrine growth factor produced by the EBV-infected lymphoblastoid cell line (V). Gel filtration over P-2 (Biorad).

**EBV-IMMORTALIZED B CELLS
FAIL TO SECRETE IL-6**

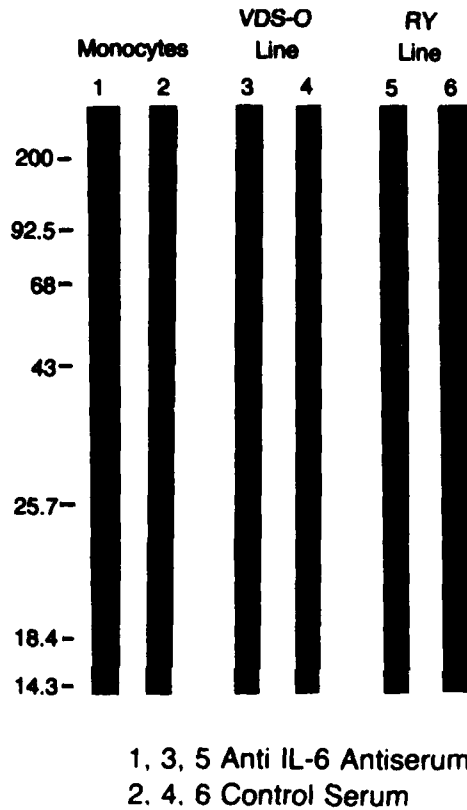


Figure 8. EBV-immortalized cells from two lymphoblastoid cell lines fail to produce immunoprecipitable IL-6. ³⁵S-labeled supernatants of LPS-activated monocytes and of two lymphoblastoid cell lines were subjected to immunoprecipitation with either a control rabbit serum (lanes 2, 4 and 6) or anti-IL-6 rabbit serum (lanes 1, 3 and 5). The immunoprecipitates were analyzed by electrophoresis under reducing conditions and autoradiography.

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